# Puncturing of lyophilized tissue engineered vascular matrices enhances the efficiency of their recellularization

Ksiazek, Agnieszka A ; Frese, Laura ; Dijkman, Petra E ; Sanders, Bart ; Motta, Sarah E ; Weber, Benedikt ; Hoerstrup, Simon P

Abstract: Data on in vitro engineered "off the shelf" matrices support the concept of endogenous cellular repopulation driving the graft's remodeling via immune-mediated response. This seems important to further accelerate the cell reconstitution and may play a crucial role when mononuclear cells are used. Nevertheless, studies on decellularized xenogeneic grafts showed only limited host cell repopulation post-implantation. This study aims at a systematic comparison of reseeding methods (dripping, injection, bathing in a cell suspension and combined puncturing-dripping method) to define the most efficient technique enhancing recellularization of tissue engineered vascular matrices (patches, vessels, small diameter and standard size valves) prior implantation. The constructs were analyzed histologically, biochemically and biomechanically. Various preconditioning treatments (wet, lyophilized and air-dried) combined with reseeding methods demonstrated the highest cell loading efficiency, despite applied crimping and flow stress, of lyophilization followed by puncturing-dripping technique. This novel seeding method allows for an efficient, time saving graft reseeding that can be used within a one-step cardiovascular clinical intervention. STATEMENT OF SIGNIFICANCE The concept of living tissue engineered, self-repairing, autologous cardiovascular replacements, was proposed alternatively to existing synthetic/xenogeneic prostheses. Recent studies in animal models demonstrate faster in vivo recellularization after grafts pre-seeding with cells prior implantation. Pre-seeded cells hold either, the ability to differentiate directionally or attract host cells, crucial for graft integration and remodeling. It is unclear, however, how efficient the pre-loading is and how well cells withstand the flow. The study presents a systematic overview on cell loading techniques of different cardiovascular constructs, tested under static and dynamic conditions. Comparison illustrates a significantly higher efficiency of cells loading in lyophilized tissues punctured before their standard seeding. This technique may beneficially accelerate remodeling of cardiovascular grafts in further in vivo studies.

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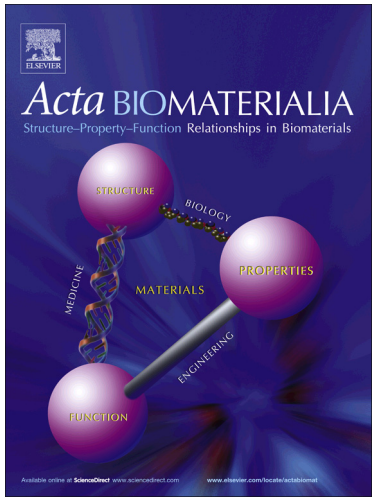


Full length article

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**Puncturing of lyophilized tissue engineered vascular matrices enhances the efficiency of their recellularization.**

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**Abstract**

Data on *in vitro* engineered "off the shelf" matrices support the concept of endogenous cellular repopulation driving the graft's remodeling via immune-mediated response. This seems important to further accelerate the cell reconstitution and may play a crucial role when mononuclear cells are used. Nevertheless, studies on decellularized xenogeneic grafts showed only limited host cell repopulation post-implantation.

This study aims at a systematic comparison of reseeding methods (dripping, injection, bathing in a cell suspension and combined puncturing-dripping method) to define the most efficient technique enhancing recellularization of tissue engineered vascular matrices (patches, vessels, small diameter and standard size valves) prior implantation. The constructs were analyzed histologically, biochemically and biomechanically. Various preconditioning treatments (wet, lyophilized and air-dried) combined with reseeding methods demonstrated the highest cell loading efficiency, despite applied crimping and flow stress, of lyophilization followed by puncturing-dripping technique.

This novel seeding method allows for an efficient, time saving graft reseeding that can be used within a one-step cardiovascular clinical intervention.

Key words: tissue-engineering, cell seeding techniques, cardiovascular grafts, recellularization, trans-catheter delivery

## 1. *Introduction*

There is a tremendous need for cardiovascular replacement constructs such as patches, vessels or valves for surgical correction of failing native tissues due to congenital disorders or degeneration processes [1]. Current clinically used materials for soft tissue repair are either non-degradable synthetic grafts or fixated tissues from xenogeneic origin and therefore they are inherently associated with progressive dysfunctional degeneration, risk for disease transmission and lack of regenerative capacity [2]. These drawbacks limit their broader use particularly in younger patients, who subsequently require several graft-related reoperations throughout the time of their growth [3]. Other alternatives include the use of bioabsorbable synthetic or naturally derived vascular grafts such as based on poly-caprolactone with 2-ureido-4[1H]-pyrimidinone [4, 5]. Despite their promising design only a trial clinical data is available questioning their further durability and functionality. Moreover, the complex "classical" tissue engineering approach was suggested, involving multistep procedures of cell harvest and expansion, scaffolds seeding, *in vitro* culture in bioreactors, defining time-critical moment of implantation of the living engineered grafts, and requiring high logistical and financial efforts. This lead to a novel non-xenogeneic tissue replacement material development, based on decellularized homologous tissue engineered matrices that do not require a human or animal starter tissue [6]. A human cell source, obtained in a safe, well-established and controlled way, is used for the production of these constructs and there is no limitation in regard to their availability, size nor risk for a disease transmission. The decellularization process enables their off-the-shelf use and longer storage making them highly attractive in the clinical setting. Pre-clinical animal studies demonstrated that the repopulation of these tissue replacements was faster in comparison to decellularized xenogeneic/allogeneic matrices from human/animal starter origin [7, 8], most likely due to their less mature collagen network. Still, observed rapid repopulation might be species and material dependent, and in the clinical trials decellularized xenogeneic- and allogeneic tissues have shown so far only sparsely endogenous cell infiltration [9-11].

Adequate and fast resorption of the acellular matrices (debris clearance, [12]) is important for three dimensional reorganization and graft integration meaning adhesion of endothelial cells, infiltration of smooth muscle cells and fibroblasts, what is regulated by the secretion of cytokines and chemokines [13]. Numerous pre-clinical trials, following this concept, used bone marrow mononuclear cells (MNCs) for reseeding decellularized grafts before implantation in order to induce such beneficial immunological response faster [14-17]. Personalized "off the shelf" tissue engineered matrix pre-seeded with MNCs could be implanted in a one-step-intervention, comprising the sample harvest, cell isolation, loading of the cells into the replacement tissue and implantation, all performed during only one surgery [16]. This approach is also more attractive in terms of the clinical setup as it circumvents costly and highly logistical production steps. However, the successful loading of the cells into the decellularized matrix is challenging, mainly due to still fairly tight, mature collagen network preventing good cell penetration. Although the formation of a surface layer of endothelial cells is often achieved [18-20], penetration of the acellular matrix requires more sophisticated strategies [21] and in most cases long *in vitro* culture time [18, 22, 23]. Therefore, in order to be able to perform a one-step-procedure of an autologous pre-seeded tissue replacement, an adequate cell loading technique prior to implantation has to be established.

In this study, different tissue engineered vascular matrices (TEVaM) and several seeding techniques as well as seeding conditions were compared in order to define the most optimal and successful protocol for matrices cell loading prior implantation. Additionally, the influence of the chosen method on mechanical stability of the construct was investigated and the stability of the loaded cells verified – both after simulated trans-catheter delivery and application of the flow stress.

## 2. *Materials and Methods*

To define the most optimal protocol for cell loading of TEVaM, different tissue engineered PGA/P4HB (polyglycolic acid/ poly-4-hydroxybutyrate) based constructs (seeded with ovine fetal mesenchymal stem cells) were cultivated according to previously published protocols [6, 8, 24], decellularized [6] and reseeded with MNCs using several cell loading methods and different seeding conditions.

### 2.1 Production of tissue engineered vascular matrices (TEVaM).

Comparison of cell loading methods was performed on four different TEVaM including: patches, vascular grafts, small diameter and standard size valves (Table 1).

Firstly, patches were used as a simple model of vascular constructs. They were used to investigate the most optimal cell seeding method from three techniques compared - each tested with three different preconditioning treatments. The most promising cell loading techniques and preconditioning treatments were further compared on more advanced constructs, meaning tissue engineered vascular grafts (TEVG) and tissue engineered valves (firstly small diameter and then standard size ones). The second ones were additionally tested for mechanical matrix stability, trans-catheter delivery and influence of the flow applied after reseeding.

*Preparation of TEVaM - production step 1.*

All scaffolds were produced from nonwoven polyglycolic acid meshes (PGA, thickness 1.0 mm (for patches and standard size valves) or 0.5 mm (for TEVGs and small diameter valves); specific gravity 70 mg/cm$^3$; Cellon) coated by dipping in the biologically derived rapidly degradable biopolymer P4HB (1.75%, Tepha Inc.) in Tetrahydrofuran (Fluka). After solvent evaporation, physical bonding of adjacent fibers and continuous coating was achieved.

- For the production of the tissue PGA/P4HB patches (n=90), constructs (25x5mm) were glued, using Polyurethane (15% PU in tetrahydrofuran), into a metal rings to avoid retraction of the tissue strips during the culture.
- For the production of TEVGs (n=36) PGA scaffolds were integrated into radially self-expandable nitinol stents (length=10 mm; OD=12 mm when fully expanded at 37°C; OptiMed) by using simple interrupted sutures (Polypropylene; Ethicon)
- For the production of small diameter valves (n=7) trileaflet PGA scaffolds were integrated into radially self-expandable nitinol stents (length=10 mm; OD=12 mm when fully expanded at 37°C; OptiMed) by using both continuous and simple interrupted sutures (Polypropylene; Ethicon).
- For the production of standard size tissue engineered heart valves (TEHV) (n=4) trileaflet PGA scaffolds were integrated into radially self-expandable nitinol stents (length=40 mm; OD=30 mm when fully expanded at 37°C; PFM, Germany) by using both continuous and simple interrupted sutures (Polypropylene; Ethicon).

All constructs were sterilized by washing in 70% ethanol solution for 2h followed by UV exposure (overnight). Subsequently they were washed in sterile PBS and incubated overnight in tissue engineering (TE) medium adjusted to the cell type used for the initial seeding (supplementary data 1A and 1B) and supplemented with L-ascorbic acid 2-phosphate (0.25 mg/ml; Sigma-Aldrich) to equilibrate the scaffold before cells seeding.

*Initial seeding, cultivation and decellularization of TEVaM - production step 2.*

Isolation of ovine fetal mesenchymal stem cells (oMSC) was performed post mortem as previously described [8, 25] (supplementary data 1A-B). TEVaM were seeded with oMSC at $1{,}5x10^6$ cells/$cm^2$ density and passage $3^{rd}$ to $5^{th}$. In all cases fibrin (consisting of fibrinogen and thrombin co-solution) was used as a cell carrier [26]. Fibrinogen (10 mg/mL of active protein) and thrombin (initial thrombin concentration: 10 IU/mL) were prepared and titrated to an optimal clotting time of ~30 s by adapting the concentration of thrombin. The fibrin seeding

procedure was performed by cells re-suspension in a fibrinogen-thrombin co-solution as previously described [27].

After seeding, the patches were cultivated under static condition for 4 weeks. In contrast TEVG and small diameter valves were cultivated statically only for 7 days and then placed in an inverted-flow bioreactor system with a unidirectional diastolic pulsatile flow applied (increasingly from 1.5 mL/min to 3 mL/min flow rate) - mimicking the native cardiovascular stress conditions [24]. TEHV were placed into a Diastolic Pulse Duplicator system for culture under dynamic strains by applying increasing trans-valvular pressure (starting from 3 mmHg on the 5th day, increasing up to 15 mm Hg within the following 4 days and kept as such until the fourth week of the culture).

All TEVaM were incubated at 37°C in a humidified atmosphere (5% CO2) and TE-medium was replaced every 3-4 days. They were harvested after 28 days of a total culture time. Decellularization process was started immediately post-harvest by applying a detergent and benzonase treatment in order to create non-vital and non-immunogenic matrices as previously described [6] (supplementary data 1C). Sterile and fully decellularized TEVaM were stored in plain DMEM medium at 4°C for further proceedings (Figure 1B).

## 2.2 Preconditioning procedures and cell loading of TEVaM.

*Isolation of Mononuclear Cells (MNCs) for cell-loading testing.*

Ovine MNCs were obtained from peripheral blood (PBMNCs) and isolated according to the adapted protocol (Sigma-Aldrich Procedure 1077, Sigma Chemical Co., USA). MNCs isolation for TEVaM loading was successful and performed without complications. Briefly, a volume of approximately 200 mL of peripheral venous blood was obtained from the jugular vein of an adult sheep into a heparinized syringe (10 IU/mL) for further isolation of MNCs. All animals received humane care and the study was approved by the local ethics committee (ZH239/14 Kanton Zürich, Gesundheitsdirektion Veterinäramt) and was in compliance with the "Guide for the Care and Use of Laboratory Animals", published by the National Institutes

of Health. The mononuclear cell fraction was isolated by density gradient separation (Histopaque® 1077, Sigma Chemical Co., USA) for 30 min at 500x g using standardized protocols [28]. The isolated MNCs fraction was either cryopreserved or directly used for cell loading. Viability of cells was assessed using Trypan Blue exclusion staining (0.4%, Gibco by Life Technologies, NY, USA).

*Preconditioning of TEVaM and MNCs loading (Table 1).*

Cell loading of TEVaM was divided into two stages: I) preconditioning step and II) the actual cell seeding with its further specification (Table 1).

Three preconditioning techniques were tested for each single seeding method: TEVaM were either kept wet in a medium, lyophilized for 2 hours (FD1. 0, Heto Lab-Equipment A/S Denmark), or air-dried at room temperature for 16 hours before the cell loading. Different cell seeding techniques tested included:

- Cells dripping (standard seeding) with or without the use of fibrin as cell carrier. MNCs were suspended either in TE-medium alone or a fibrinogen-thrombin co-solution (as described above) [27] and loaded onto preconditioned TEVaM ($1{,}5x10^6$ cells /cm2). After 15 min static incubation TE-medium was added and samples were further incubated for 1 hour at 37°C.
- For bathing the preconditioned TEVaM were placed in a closed container system with 5 ml/construct of MNCs-TE-medium suspension ($4{,}5x10^6$ cells/cm2). After 15 min static incubation, samples were further incubated for 1 hour at 37°C in either static condition or under applied 2D or 3D stress (shaking).
- Cells injection was performed either with or without the use of fibrin as a cells carrier. MNCs were suspended in either TE-medium or a fibrinogen-thrombin co-solution (as described above) [27] and injected using a 29G needle at 2 locations/$cm^2$ ($1.5x10^6$ cells /cm2) into the preconditioned TEVaM. After 15 min static incubation, TE-medium was added and samples were further incubated for 1 hour at 37°C.

- Basing on the results obtained from patches, a novel seeding technique combining puncturing and dripping was included and used for more advanced TEVaM in order to overcome the manually challenging injection method. For this purpose, tissues were punctured superficially 25 times/$cm^2$ with 29G needle and seeded with the dripping method without the use of fibrin. As a result, the porosity of a dense surface layer of the constructs' extracellular matrix was artificially and intentionally increased in order to ease the cell entrapment. For puncturing of the standard size TEHV, a custom-made puncture device was developed (Figure 1A), enabling operator independent partial matrix perforation and guaranteeing a standardized puncture pattern, following the exact belly shape of the TEHV [29]. It also allows a strictly defined diameter and depth of the perforations. After puncturing the MNCs were suspended in TE-medium and dripped over TEVaM ($1.5x10^6$ cells /cm2). After 15 min static incubation, TE-medium was added and samples were further incubated dynamically (with applied flow) for 1 hour at 37°C.

Only the most promising preconditioning steps and most effective cell loading techniques were further tested in more advanced groups of TEVaM (Table 1). Thus, after initial evaluation of all the techniques performed on patches (and compared to the unseeded control group n=3), TEVGs were either wet or lyophilized when seeded via dripping, injection or puncturing and dripping methods. The air-drying preconditioning step and seeding via bathing were eliminated from further analysis based on the results obtained with patches. In the further analysis also the seeding method via injection was excluded from evaluation in valvular constructs.

While patches were predominantly incubated in the static condition post reseeding, other TEVaM had a stress flow applied following their previously established production steps. TEVGs were incubated with pulsatile flow applied and the results were compared to the control static culture of TEVGs (n=6). Small diameter valves (Figure 1C) were incubated in a similar fashion with some modifications including fluorescence analysis performed and trans-catheter delivery simulation (2.3 and 2.4 section) and were compared to the unseeded,

non-punctured control (n=1). Standard size TEHV were tested, post seeding and simulated trans-catheter delivery, in previously described Diastolic Pulse Duplicator systems and were compared to non-punctured control (n=1).

2.3. Simulated trans-catheter delivery of cell loaded small diameter valves and standard size TEHVs.

In order to investigate MNCs preservation in TEVaM after trans-catheter delivery and flow application, simulated crimping was performed and further tested to assess the success of the most promising cell loading methods. Therefore, small diameter valves (n=7) and standard size TEHVs (n=3) were loaded with MNCs using different seeding methods (all without the use of fibrin; Table 1), incubated for 1 h and subsequently crimped for 10 min at room temperature down to 15 F (5 mm OD; small diameter valves) or 30 F (10 mm OD; TEHV) (Figure 1D-E; using a customized crimping machine; Edwards LifeSciences, Inc.), mimicking the setting for delivery device (catheter) prior to surgical implantation. Subsequently, the cell-loaded valves were deployed in medium and transferred back into the flow bioreactor system for 1h to investigate the cellular loss induced by crimping and post-crimping flow stress (2 mL/min flow rate).

MNCs used for loading of small diameter valves were additionally pre-labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) in order to compare and ease cell tracking pre- and post-crimping and access the cellular loss induced by the simulated trans-catheter delivery (details below). Therefore, CFSE pre-labeled MNCs (n=3), non-CFSE pre-labeled MNCs (n=3) seeded on small diameter valves and not seeded control (n=1) were compared; whereas TEHVs were all (n=3) seeded with non CFSE pre-labeled MNCs.

*MNCs* carboxyfluorescein succinimidyl ester *(CFSE) labeling before loading in small diameter valves.*

To further confirm the resistance of the loaded cells against the necessary crimping procedure and applied flow stress, MNCs were labeled with the fluorescent dye carboxyfluorescein

succinimidyl ester (CFSE) and subsequently tracked by fluorescence analysis before and after crimping and applied flow stress. Prior to cells loading onto small diameter valves, MNCs were labeled using CFSE dye at a concentration of 5 mM (CFSE, CellTrace; Invitrogen Corp.) and incubated for 15min at 37°C, 5% $CO_2$ under the light protection. Afterwards, they were centrifuged at 280 g for 10 min, supernatant was removed and cells were washed with PBS. Centrifugation step was repeated and CFSE labeled MNCs were loaded on miniaturized valves (n=3) with the according seeding methods (Table 1).

2.4. Analysis of TEVaM.

*Histological staining.*

For qualitative evaluation of both complete decellularization and the efficiency of different cell-loading methods, all TEVaM were analyzed by Haematoxylin-Eosin (HE) staining. Therefore, representative samples were fixed in 4% formalin for 12 hours, embedded in paraffin, cut into 5µm sections and stained with HE. Each sample was analyzed from 3 independent section regions and only the most representative for a group section was presented. All sections were analyzed using an inverted light microscope (ZEISS Axiovert 40 CFL and ZEISS Axioplan II; Carl Zeiss AG).

*DNA-content analysis.*

Cell loaded TEVaM were analyzed quantitatively for their total amount of DNA, as an indirect indicator for the cell number. The amount of DNA was measured using the commercially available Quant-iT™Picogreen® dsDNA kit (Molecular Probes, Invitrogen). PicoGreen® being a fluorescent probe binds dsDNA and forms a highly luminescent complex when compared to the free dye in solution. The amounts of DNA per samples were calculated and expressed per mg dry weight of tissue. The biochemical measurements were presented as mean ± standard deviation of the tested samples.

*IVIS® fluorescence crimping analysis.*

Using the IVIS® Imaging System (PerkinElmer Life Sciences, Inc.), CFSE-labeled miniaturized valves (n=3) and their non CFSE-labeled corresponding controls (n=3), were scanned before crimping and the fluorescence (counts) was determined. For detection of CFSE-labeled MNCs, a band-pass filter from 465 nm and a long-pass filter over 520 nm for excitation and emission light, respectively, were used. The fluorescence was detected by CCD camera. The data was analyzed using the Living Image software (Living Image 3.2; PerkinElmer Life Sciences, Inc.). After the pre-crimping analysis constructs were crimped for 10 min down to 15 F (5 mm), and returned to the pulsatile flow bioreactor for further incubation (1 h). Post flow stress IVIS® scan was repeated and results compared with the pre-crimping analysis. Additionally, one non-reseeded decellularized miniaturized valve was used as an IVIS® background signal control (n=1).

*Biomechanical Analysis*

In order to analyze the biomechanical stability of the decellularized TEHV after the lyophilization and/or puncturing, biaxial tensile tests were performed on the leaflets of decellularized TEHVs. Therefore, samples were taken per treatment group from each valve: wet TEHV after being punctured (n=3 leaflets), lyophilized and punctured TEHV (n=3 leaflets) or TEHV left untreated (wet) as a control (n=3 leaflets). The mechanical properties of the leaflets were determined using a bi-axial tensile tester (BioTester, 5N load cell; CellScale, Waterloo, Canada in combination with the LabJoy program; V7.05, CellScale). Prior to testing, tissue thickness was measured by using an electronic caliper (CD-15CPX, Mitutoyo, Japan). The samples were stretched equibiaxially in both the radial and circumferential direction up to 20% strain, at a strain rate of 100% per minute. After stretching, the samples recovered to 0% strain at a strain rate of 100% per minute, followed by a rest cycle of 54 seconds. Prior to measuring the final stresses, samples were preconditioned with 5 of these cycles. A high-order polynomial curve was fitted on each individual data set in both the radial and

circumferential direction. The stiffness of the tissue was represented by the tangent modulus and was defined as the tangent to the fitted polynomial curve at 20% strain and presented as the mean ± standard deviation of the tested samples.

### *3. Statistical analysis*

Biochemical DNA measurements are presented as mean ± standard deviation. For statistical comparison of the DNA results a Mann-Whitney-U test was performed (GraphPad Prism 5, GraphPad Software Inc., CA, USA) for matching variables and for multiple comparisons between the variables a one-way ANOVA (Dunnett's Multiple Comparison Test) was performed. Each group of the results was tested for outliers using Grubb's test and found values were excluded from further statistical analysis. For biomechanical analysis, treatment groups were compared to the control group in both radial and circumferential direction by using a non-parametric ANOVA test, combined with a post-hoc Dunn's multiple comparison test. Results were considered significant for a p-value $< 0.05$ (95% confidence interval). For multiple comparisons a Bonferroni correction was applied and a p-value $<0.01$ was considered significant (99% confidence interval).

## *4. Results*

TEVaM were analyzed starting from less to more advanced constructs and results were summarized in four main statements, based on all the analysis performed per group of constructs, and compared with each other and with the controls.

### 4.1 Statement 1: better cell loading is achieved by dripping or injection without the use of fibrin.

Haematoxylin-Eosin staining of control groups proved a complete decellularization of all TEVaM constructs before MNCs loading. In case of patches, the HE staining demonstrated cell penetration into wet, air-dried, as well as lyophilized tissues when the *dripping* or *injection* seeding techniques were applied (Figure 2). The *bathing* resulted only in attachment of few cells, what was confirmed with a quantitative biochemical DNA-PicoGreen® assay, and no cells were visible on histological analysis. This technique was least favorable of all tested, presenting the smallest cell holding ability no matter the preconditioning used, thus was excluded from further analysis (Figure 2 and 3 A-C). The DNA assay for decellularized patches, post MNCs loading with variable seeding methods, demonstrated the highest DNA concentration (µg/mg of dry tissue, reflecting cellularity level) predominantly in the *injection* group (without fibrin) for all preconditioning techniques (wet, air-dried and lyophilized), noting that it was significantly more effective for the air-dried patches in comparison to the lyophilized ones ($p=0.0033$). Similar results were obtained for the *dripping* method, in exception to the wet preconditioning group, where cellularity was significantly lower in comparison to the air-dried ($p=0.0024$) and lyophilized ($p=0.0003$) groups (Figure 3 A-C).

The use of fibrin as a cell carrier resulted mainly in superficial, non-cross-linked cell-fibrin layer "trapped" on top of the tissue, without cellular penetration into the deeper layers (Figure 2). On the other hand, the patches seeded without the use of fibrin showed cellular penetration also into the tissue core. For both, *injection* and *dripping* groups, seeding without the use of fibrin resulted in significantly better cellularity post MNCs loading (*dripping*: $p=0.0275$; *injection:* $p=0.0001$) (Figure 3C).

Air-drying as a preconditioning step affected the tissue integration resulting in random creation of holes in the tissue matrix (visible on HE histology analysis, Figure 2), thus, despite the promising DNA assay results, it was excluded from further analysis.

4.2 Statement 2: enhanced cell loading, despite applied flow stress, is achieved in decellularized TEVGs by the *injection* – or the modified *dripping* method with prior tissue *puncturing*.

Following the promising results obtained with the *injection* method, a more automatized and easily repeatable, operator independent method of *puncturing and dripping* was also tested. Both seeding techniques, when evaluated with HE histological staining, confirmed MNCs loading and cells withstanding the flow stress applied, independently from the preconditioning procedure used (wet or lyophilized). In contrast, no cells were detected post cell seeding by *dripping* technique alone (without prior puncturing) (Figure 4).

Unlike in the histological observations, the biochemical DNA- PicoGreen® assay detected non-significantly different amounts of DNA concentrations for all applied seeding methods and preconditioning procedures when compared with each other. The highest cellularity was achieved with the lyophilized *puncture-dripping* method (Figure 5).

4.3 Statement 3: lyophilization and the *puncturing-dripping* - the most successful cell loading technique for TEVaM allowing cells to maintain in a tissue core after simulated trans-catheter delivery and applied flow stress.

The quantitative DNA-PicoGreen® analyses revealed significantly higher number of cells (highest DNA concentration corresponding to cellularity level) after crimping and exposure to flow when TEVaM (small diameter valves and TEHVs) were lyophilized and *punctured* before being seeded by the *dripping* method ($p<0.0001$ when compared to the lyophilized *dripping* group; $p=0.0011$ when compared to the wet *dripping* group) (Figure 6A). Dripping method alone was only significantly better when the wet preconditioning was used ($p=0.0084$), but was still less efficient in comparison to the lyophilized punctured matrices.

Further quantification of cellularity loss, performed on small diameter valves with fluorescence detecting IVIS® system, revealed the highest post-seeding signal (fluorescence intensity reflecting the cellularity level) in the lyophilized *dripping* group and the lowest signal for the wet *dripping* method. Nonetheless, once simulation of catheter delivery (crimping) and flow stress were applied, the highest cellular loss of 55% was noted for the lyophilized *dripping* method. For the other two methods it equaled 40% of signal intensity (cellularity) loss. Thus overall, the highest fluorescence signal withstanding simulated catheter delivery and flow application was again noted for the lyophilized, *puncturing-dripping* group, further confirming its superiority over the other methods (Figure 6B-C).

Dynamically cultured decellularized TEHVs were used to ultimately verify the *puncturing-dripping* method of lyophilized matrices after crimping and flow application.

The HE staining for TEHVs repeatedly demonstrated the superiority of lyophilization and *puncturing dripping* method by significantly better cell infiltration within the tissue core (Figure 7B-C1). The regular pattern and depth of puncturing (performed with operator independent custom made puncturing device) enabled homogenous cell infiltration into the tissue core and increased their resistance against crimping and flow (Figure 7C1,C2). Cells maintenance within the TEHVs after the simulated crimping and flow application (with only minor non-significant cell loss) was also confirmed by DNA- PicoGreen® analysis (Figure 7A).

4.4. Statement 4: puncturing the lyophilized TEVaM before cell-loading did not change the mechanical stability of the tissue.

Biaxial tensile tests were performed to assess the effect of the different treatment options on the mechanical properties of TEHVs. Tangent moduli in radial and circumferential direction were calculated from both treatment groups (wet punctured and lyophilized punctured) and compared to the control group (wet not punctured). Based on these results, the treated tissue groups were not significantly different from the control group confirming the mechanical

stability of the construct despite the performed lyophilization and puncturing procedures (Figure 7E-F).

***5. Discussion.***

The study broadly compares different approaches and available techniques used in recellularization of decellularized tissue engineered vascular matrices (TEVaM) prior to their simulated implantation. Once established, such rapid and optimal seeding protocol (with the use of patient autologous cells) can be used in variety of "off-the-shelf" tissue replacement products [6], allowing cell survival and their endurance in simulated trans catheter delivery. Optimally, such seeding method should allow for a one-step surgical intervention [16, 30] focusing on both a less-invasive cell source and cell loading that does not require time consuming active infiltration. The present study compared and tested several pretreatments and seeding conditions *in vitro* on simple to complex tissues (such as heart valves) revealing that the highest post seeding cellularity, withstanding the simulated trans catheter delivery and flow stress, can be achieved by lyophilization and a novel *puncturing-dripping* method, without the use of fibrin as a cell carrier. Such a seeding technique is uncomplicated, operator independent, does not require a prolonged incubation time and can be performed at a single intervention with patients' autologous cells obtained in non- or minimally invasive way.

As the clinical relevance of living replacement tissues is large, many groups have investigated methods to enhance cell loading for decellularized grafts. Such "repeated seeding" of autologous cells on cell-free matrix prior to the *in vivo* implantation, proved to prevent graft stenosis, calcification as well as valvular insufficiency and cusps thickening [31-34]. Recent findings indicate that once implanted, TEVaM undergo an inflammatory-driven process of "debris clearance" and errors in this mechanism can negatively influence their regeneration [12]. Many pre-clinical studies using MNCs for grafts pre-seeding, were comparing the cytokine release rather than cell viability [35]. Cytokine release (by a viable or apoptotic cells) is triggering an in situ inflammatory response leading to faster remodeling. Thus an important factor is to be able to keep the pre-seeded cells in the desired place of faith. MNCs, even after their apoptosis, are considered to be a key point of triggering the inflammatory response in wound healing, being beneficially associated with active tissue formation [36]. Moreover, such controlled cellular attraction and adequate matrix remodeling are important

determinants for long-term function and stability of the graft [37]. This process is directly involved in neovascular tissue formation [38].

MNCs have the potential to differentiate into many different cell types *in vivo* [39] and are known for their paracrine effects that can guide the repopulation and remodeling responses [38]. The enhancement of such endogenous repopulation might be of a large clinical relevance for both tissue engineered matrices as well as decellularized xenogeneic/allogeneic tissues, eventually leading to a successful clinical translation of the novel off-the-shelf concept. Therefore, MNCs were the cell line of choice for cell loading experiments in the current study. Moreover, such accelerated remodeling after recellularization means also faster graft endothelialization, what is particularly important in patients with anticoagulation therapy contraindication, even if just needed at the initial phase post implantation. The same stands for the patients with debilitated healing, such as those under steroid treatment or with metabolic disorders (i.e. diabetes mellitus).

Currently used re-seeding methods often enable a formation of a surface layer of endothelial cells [18, 40], but penetration of the cells into the deeper tissue layers mostly relies on prolonged *in vitro* culture time [18]. It has been hypothesized that the limited cell penetration is most likely due to the thick collagen network in mature tissues [41]. Therefore, to enable immediate cell loading into the core of the decellularized matrices, a more sophisticated strategy allowing penetration of the dense collagenous surface is required. This problem was mainly addressed in the studies investigating the repopulation of the xenogenic/allogeneic decellularized grafts. One of these concepts was based on a laser-induced increment of the matrix porosity [42] and was tested long term *in vivo*. The results showed significantly better reconstitution with host cells and faster remodeling with signs of neovascularization in the highly porous laser treated group, in comparison to the untreated control.

Following these promising results, the current study aimed at comparison and establishment of a most effective MNCs loading method in decellularized TEVaM that were produced from

biodegradable materials with no involvement of xenogeneic/allogeneic tissue. Several tests were performed on multiple TEVaM to choose the best combination of the techniques. Despite the presumably different architecture (porosity) of post preconditioned TEVaM, unification of the seeding techniques for all of the conditions, resulted in still conclusive and comparable results. Additional studies can focus more on the base tissue characterization what was not the focus of this work.

An influence of air-drying and lyophilization prior to seeding was tested on the tissue-engineered patches showing some superiority over the wet condition. Despite the highest cellularity obtained for patches in the air-drying group the preconditioning process was more time consuming than other treatments, what also increases the infection risk. Moreover, the slowly progressing air-drying resulted in uncontrolled formation of hole-like artifacts increased in numbers per tissue construct area comparing to other preconditioning methods (histological evaluation, Figure 2). Such affected tissue architecture could result in mechanical instability and dynamic failure once being implanted and therefore it was excluded from further evaluation on more complex constructs. Only lyophilization (snap drying) and wet preconditions were tested further.

Bathing the tissues in cellular suspension resulted in insufficient cell attachment both proven by histology and DNA analysis (Figure 2 and 3A,C). It required a larger volume of medium and much higher cell numbers for loading in comparison to the other methods. Consequently, it would also require a larger patient sample size, with low efficiency of cell use, as a result of cellular loss/waste due to inadequate cell attachment and cell washout. This method was thus not further investigated, as it was considered being ineffective, demanding high cellular numbers and costly.

The use of fibrin as a cell carrier was suggested to help the cells to distribute homogenously and prevent the cells from detachment during crimping and implantation. Even though the hypothesis was proven to be true [26], only superficial cell coverage was obtained when fibrin was used with no cellular penetration into the deeper layers. Neither injection nor dripping

method enhanced cell infiltration when fibrin was used and for the majority of groups histological evaluation confirmed the results of DNA analysis showing only superficial cells entrapment in the TEVaM (Figure 2 and 3A-C). It can be concluded that, despite the seeding method used, significantly better cellular infiltration is achieved without the use of fibrin. Also given the fact that fibrin usage in an additional, time consuming step, potentially holding higher infection risk it was excluded from further analysis.

Vincentelli and co-workers demonstrated the feasibility of cell injection directly into the leaflets of decellularized xenogeneic heart valves [21], therefore, this seeding method was also included in the study protocol. The method proved cell penetration into the matrix both for patches and TEVGs seen on histology and DNA analysis (Figure 2-5). However, alike the previous study, also in the current experiment cells were not homogeneously distributed but locally delivered creating "cluster-like-formations" [21]. Moreover, the handling of the cell injection into TEVaM, was technically challenging (particularly for tissues of more complex geometry) and operator dependent. This was mainly due to the insufficient tissue thickness, small injection volume and limited number of injection sites. Additionally, a histological evaluation of the injection method was highly dependent on the sectioning place and its accuracy on targeting the injection site. Driven by the promising results of this technique and avoiding its reproducibility difficulty – a modified method of dripping with prior novel tissue puncturing was developed. Moreover, to further ease the seeding process using this technique in TEHVs, a novel puncturing device was established with a future prospect of the process' full automatization and electronic control. Again, seeding non-punctured tissues resulted mainly in a surface cell layer, while dripping the cell suspension onto punctured and lyophilized tissues demonstrated significantly better cellular integration into the core of the decellularized TEVaM, predominantly in the more geometrically complex constructs (valves). A discrepancy was only observed between post-seeding DNA content analysis and histological evaluation for wet preconditioning and dripping versus punctured-dripping methods in TEVGs. Namely better results (not significant) were achieved for the wet dripping alone method in DNA analysis (Figure 5) when compared to the wet puncturing-dripping

group what, however, had no confirmation in the histological evaluation (Figure 4). Such difference may be related to the cell allocation at the tissue surface layer for the wet dripping group with no deeper cell penetration given denser ECM (extracellular matrix) architecture of TEVGs, yet again histological evaluation might be related to the slide-sectioning locum accuracy. Comparing the results obtained in other TEVaM, a conclusion can be made that for the wet tissue the dripping method alone can be a preferable one. Nevertheless, significantly better cellularity is obtained when tissue undergo lyophilization and is loaded with puncturing-dripping technique, which is particularly a favorable one for more complex TEVaM (TEVGs, small diameter and standard size TEHVs). As they consist mainly of mature collagen matrices, lyophilization in combination with puncturing-dripping as a seeding method allowed for significantly better cell holding ability within the tissue than any other seeding technique tested (Figure 6A-7B-C1). It can be hypothesized, that lyophilization alongside with puncturing before seeding enabled higher cell numbers to be loaded by increasing the porosity of the dense ECM architecture of the constructs, similarly to the results obtained by Bergmeister and coworkers from the laser-perforation of allogeneic grafts [42]. Simulated catheter delivery (crimping) and flow stress applied had the smallest negative impact on cellular loss in this group what was proven in all performed tests including additionally performed fluorescence analysis with the IVIS® system (Figure 6B-C). Biomechanical biaxial test confirmed tissue stability and no significant influence of lyophilization and puncturing on the tissues (tested on TEHVs) (Figure 7E-F). This combined method allows for a homogenous cells distribution over the entire graft, even though cells are predominantly located at and around the puncturing sites what can be noted in Figure 7C1-C2. Also, when compared to the native human heart valves (aortic and pulmonary), lyophilized and punctured tissues present similar cellularity (Figure 7 C1-D2).

The main study limitation was the reliability of the method allowing the adequate measurement of the loaded cells. Histology allowed for a demonstration of cellular distribution within the tissue distinguishing whether the cells formed only a superficial layer or had penetrated deeper into the matrix. This evaluation, however, was highly dependent on

the sectioning site and thickness thus may have not truly represented the effectiveness of the method. DNA assay, on the other hand, could only quantify the amount of cells loaded, but not distinguish their location – thus only a combination of these two assessments allowed for a proper evaluation of the seeding technique used. In addition, a fluorescence analysis was performed using the pre-labeled MNCs confirming the results obtained in the other two evaluation tests. Nonetheless, IVIS® analysis was only performed additionally and on a small number of constructs thus the results from this test hold low statistical power and were considered only as additive information. Different signal intensity detected at IVIS® analysis after seeding only (despite the same number of cells seeded), can be explained by the discrepancy in tissue macroscopic morphology given different preconditioning procedures used. Even though the same cellular loss was noted for both lyophilized punctured-dripping and wet dripping groups, still better cellularity post simulated trans-catheter delivery was observed in pre-punctured lyophilized valves (Figure 6B).

To overcome the difficulties with the injection method and allow for a repetitive and - operator independent puncturing, a novel device was developed. It consisted of an insert, following in its design the exact belly shape of the heart valve leaflets, and three pushers puncturing the tissue with a defined diameter, depth, and pattern (Figure 1A). Specification of the puncturing can still be optimized and automatized in order to enhance a more equal cell distribution. It has to be noted however, that manipulation with these parameters is limited by the mechanical stability of the tissue and leaflets' thickness.

The aim of this study was to promote the current bioengineered tissue replacements towards time effective medical intervention. This would comprise the bone marrow puncture or peripheral blood sampling, cell isolation, loading of the cells into the graft, and its implantation in one surgery only [16]. Such a one-step-intervention approach is clinically attractive as it circumvents costly and highly logistical efforts due to elimination of time-consuming and infection risk holding in vitro culture time that is required at this point for cellular attachment. The presented novel cell-loading by the puncturing-dripping method, makes use of available off-the-shelf pre-treated, meaning decellularized, lyophilized tissues,

that can be seeded in the operation theatre with autologous mononuclear cells (MNCs). Lyophilization ensures a prolonged shelf life time and enables gas sterilization of the final product before cell loading. As the seeding method should fulfill the requirements for a one-step-intervention with minimally invasive implantation technique, the necessary crimping, loading into the catheter and graft deployment, were simulated in the current study. The overall time of all procedures did not extend two hours. The study proved that the loaded cells withstand such crimping and maintain within the core of the TEVaM after deployment and upon exposure to flow.

The most appropriate cell type for loading the TEVaM can vary and be application dependent. As previously mentioned, mononuclear cells (MNCs) are known for their paracrine effects, acceleration of "debris clearance" and ability to guide the repopulation and remodeling response *in vivo* [38], being the reason of using them in the present study. The superiority of the lyophilization in combination with puncturing and dripping technique, however, can also be relevant for other cell types and sources. It is hypothesized that a successful cell loading of MNCs into the core of decellularized tissue engineered vascular matrices (TEVaM) will facilitate endogenous cell infiltration via chemotaxis, what subsequently initiate the cascade of remodeling towards the native counterpart. Based on the presented promising *in vitro* results, further *in vivo* studies should follow in order to prove the faster remodeling and longer stability of cell loaded TEVaM seeded with puncturing-dripping method versus grafts seeded with other techniques. Such envisioned living tissue replacements, being able to more effectively "guide" their remodeling via programmed endogenous cell attraction and remodeling, all prepared in a one-step procedure, may revolutionize the current therapeutic tissue replacement strategies.

***6. Conclusions.***

The study introduces and proves the *in vitro* superiority of a novel cell-loading technique for TEVaM consisting of a combination of lyophilization and puncturing followed by dripping method. It enables immediate autologous cell loading into the core of decellularized engineered matrices prior their implantation, despite the dense collagen architecture of these complex grafts. Lyophilization ensures a prolonged shelf life time and enables gas sterilization of the final product. Such matrix seeded via dripping method with prior puncturing has proven an achievement of higher graft cellularity after the simulated surgical intervention consisting of crimping, trans-catheter delivery and flow application. Matrices seeded with such operator-independent and easy-to-perform technique, preserve their mechanical stability and are expected to remodel faster towards the native tissue with their long-term stability preservation once tested *in vivo*. Presented technique was successful for tissue engineered cardiovascular replacements *in vitro*, but could also be of a clinical importance for a broadly used other dense cell-impermeable grafts such as decellularized xeno- or homogeneic tissues. This requires, however, further confirmation in already on going *in vivo* large animal studies. Because tissue repair is applied in a variety of regenerative therapies, this clinically attractive cell-loading technique could contribute to the successful application of tissue engineering into the clinical setup worldwide.

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**8. Declaration of interest.**

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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**Table 1**

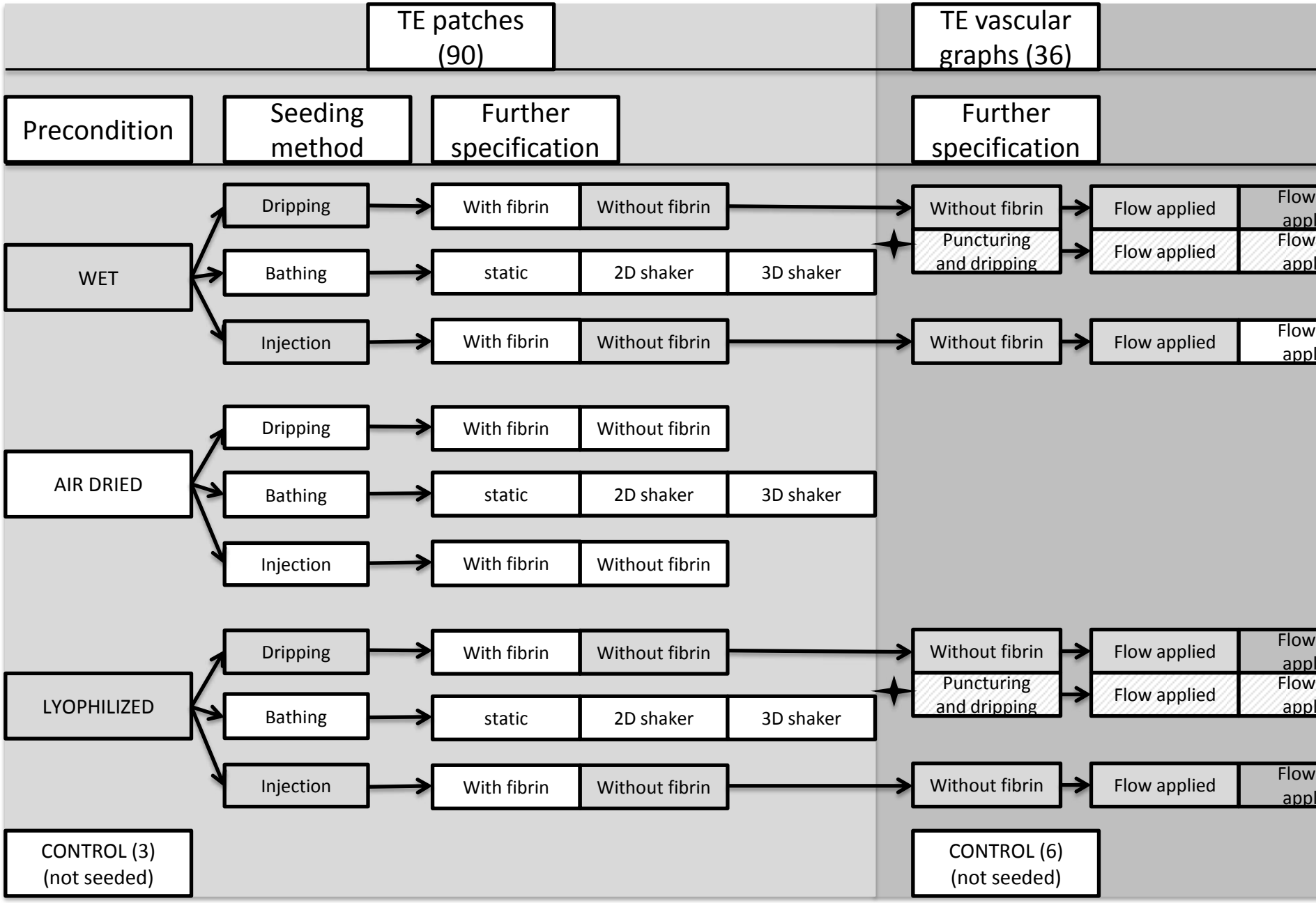

TE patches (90)
TE vascular graphs (36)
Precondition
Seeding method
Further specification
Further specification
WET
AIR DRIED
LYOPHILIZED
Dripping
Bathing
Injection
With fibrin
Without fibrin
static
2D shaker
3D shaker
Without fibrin
Puncturing and dripping
Flow applied
CONTROL (3) (not seeded)
CONTROL (6) (not seeded)

Figure 1



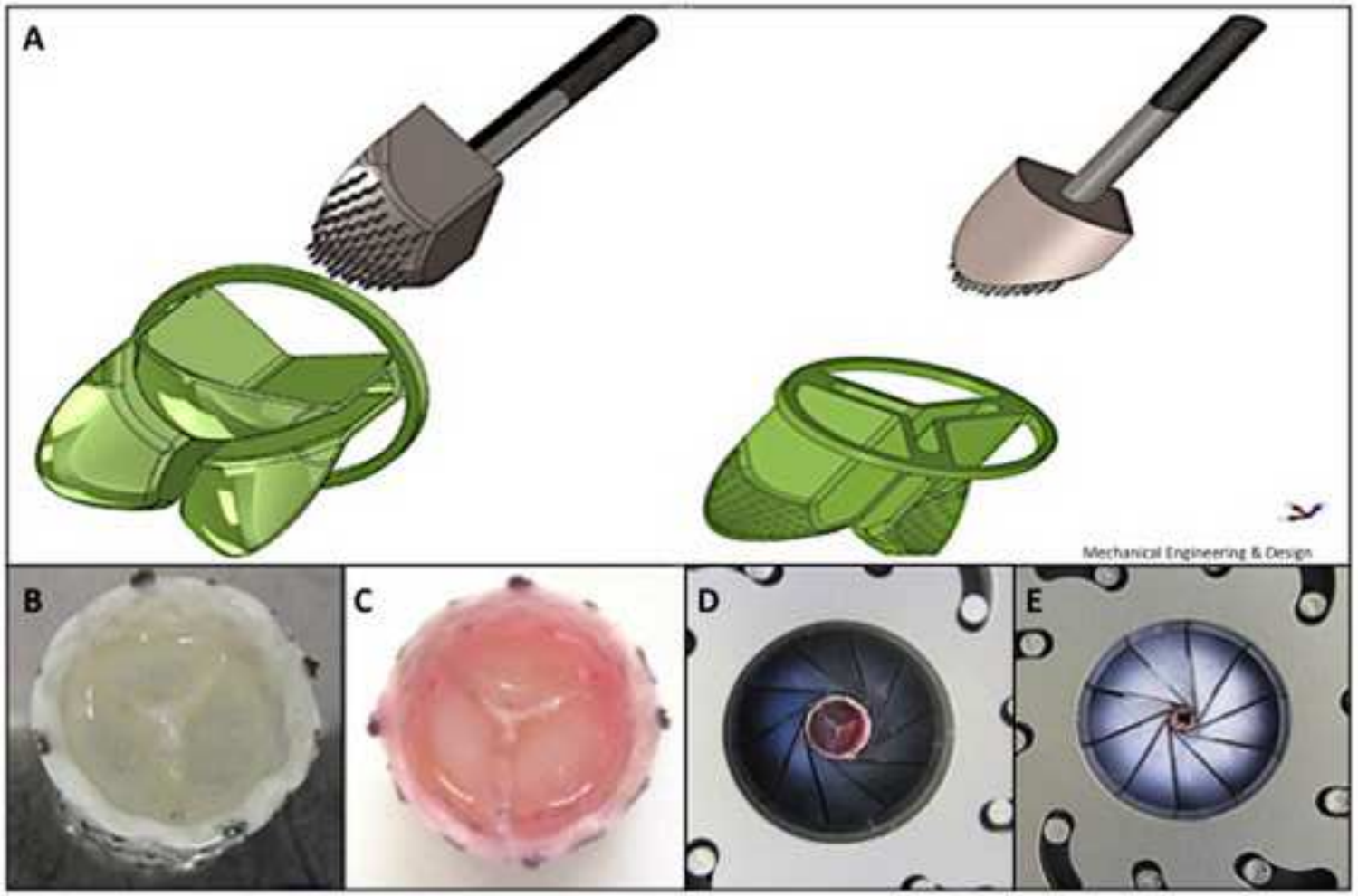


Figure 2



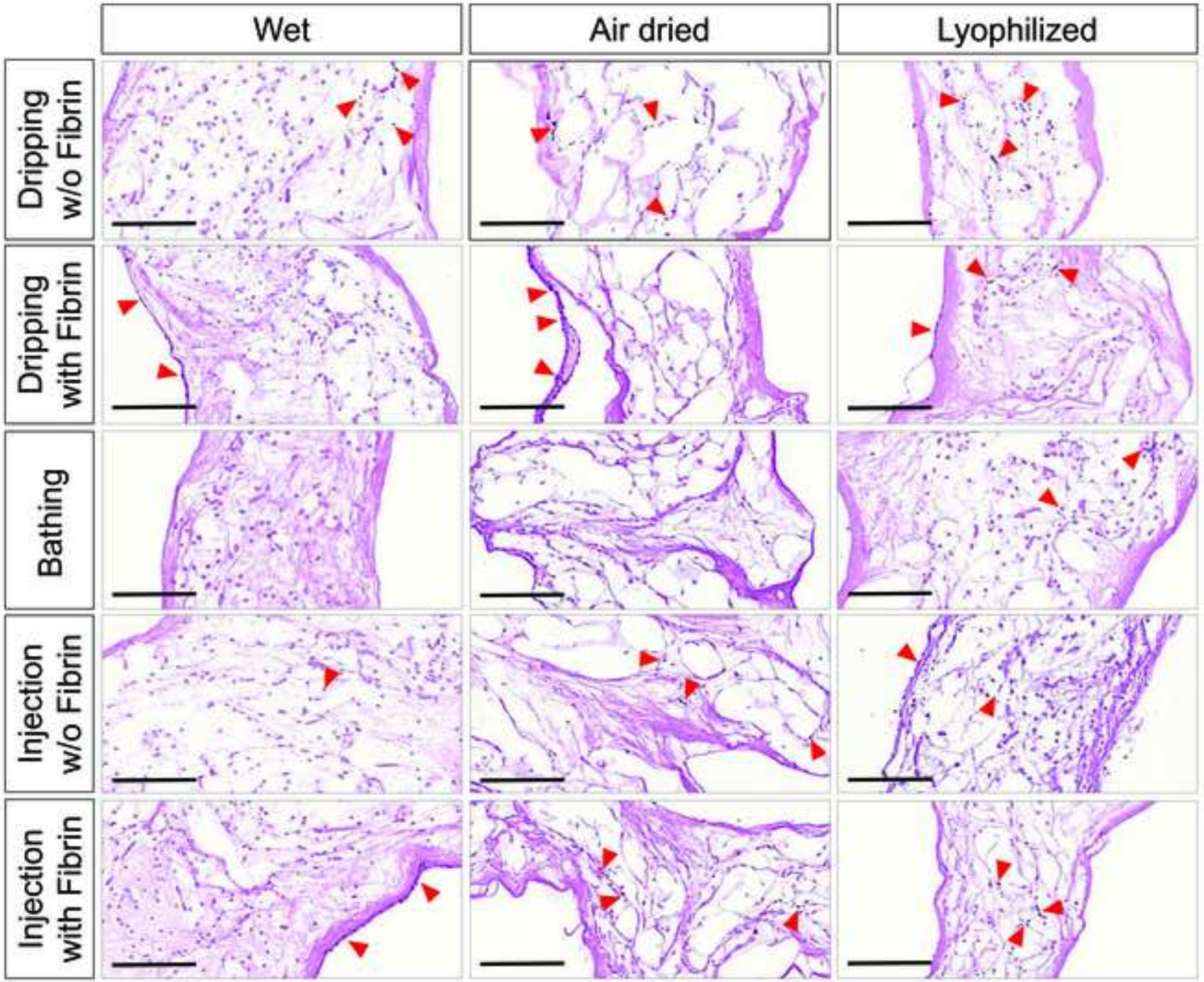


Figure 3



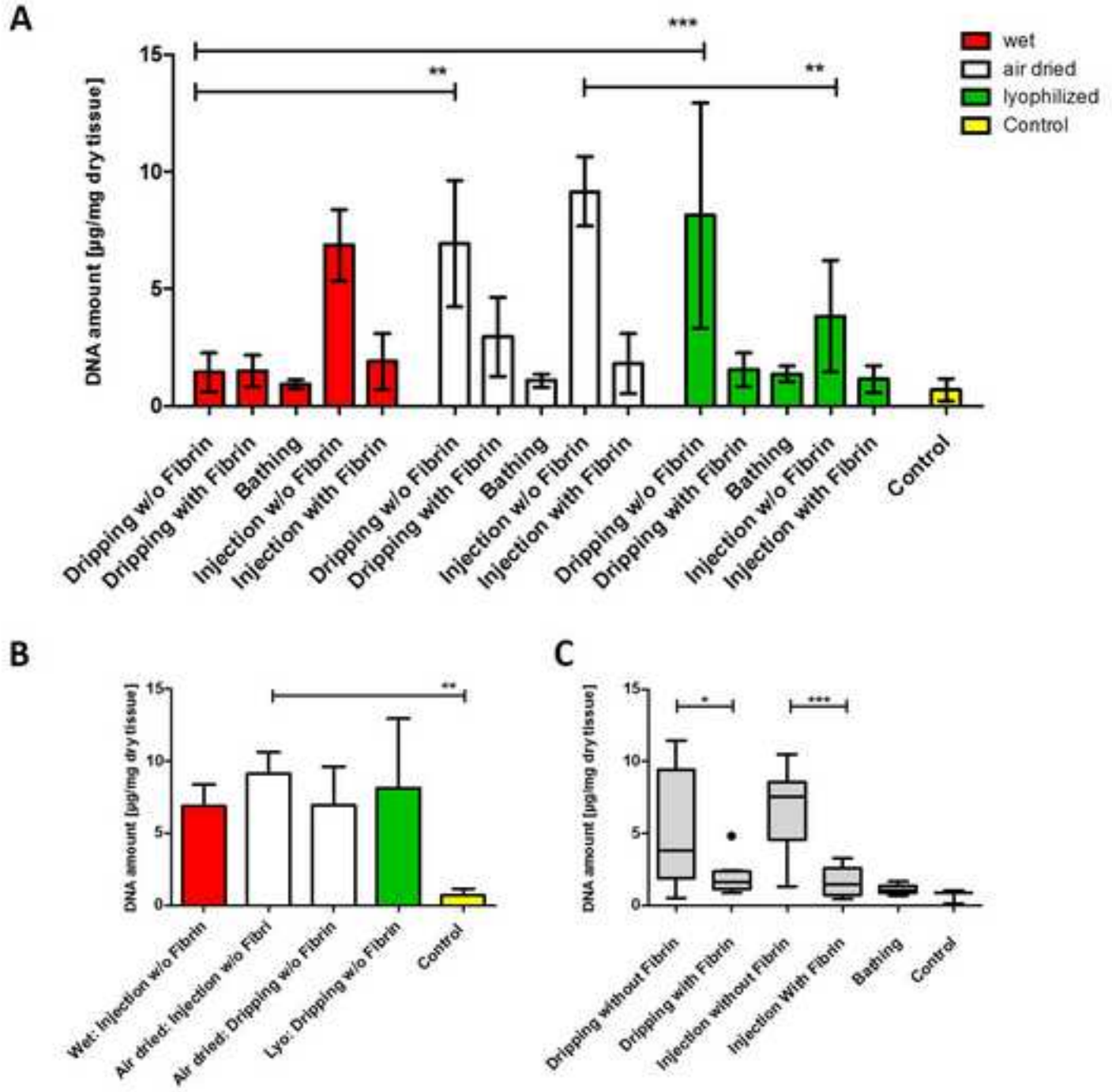


Figure 4



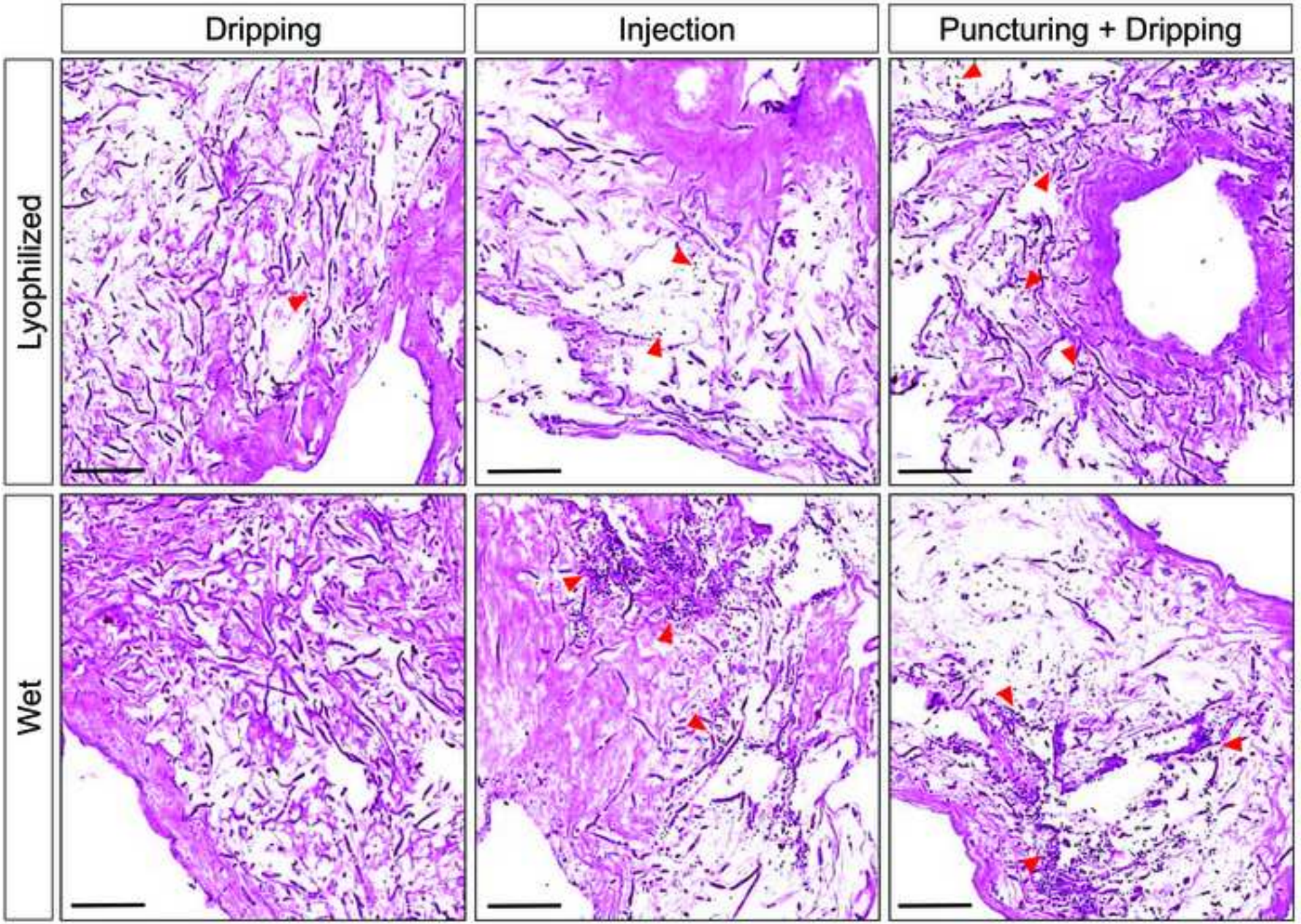


Figure 5



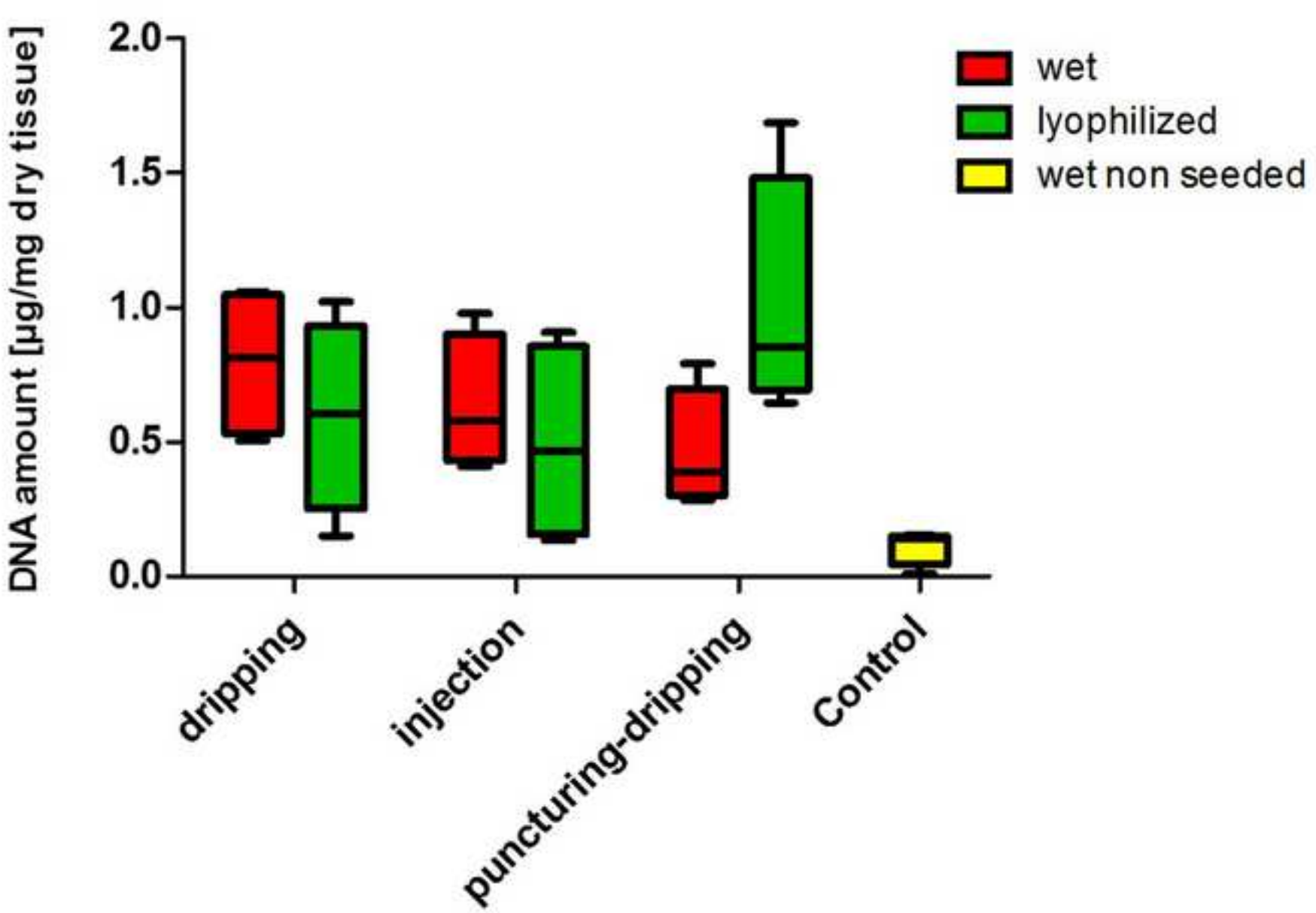


Figure 6



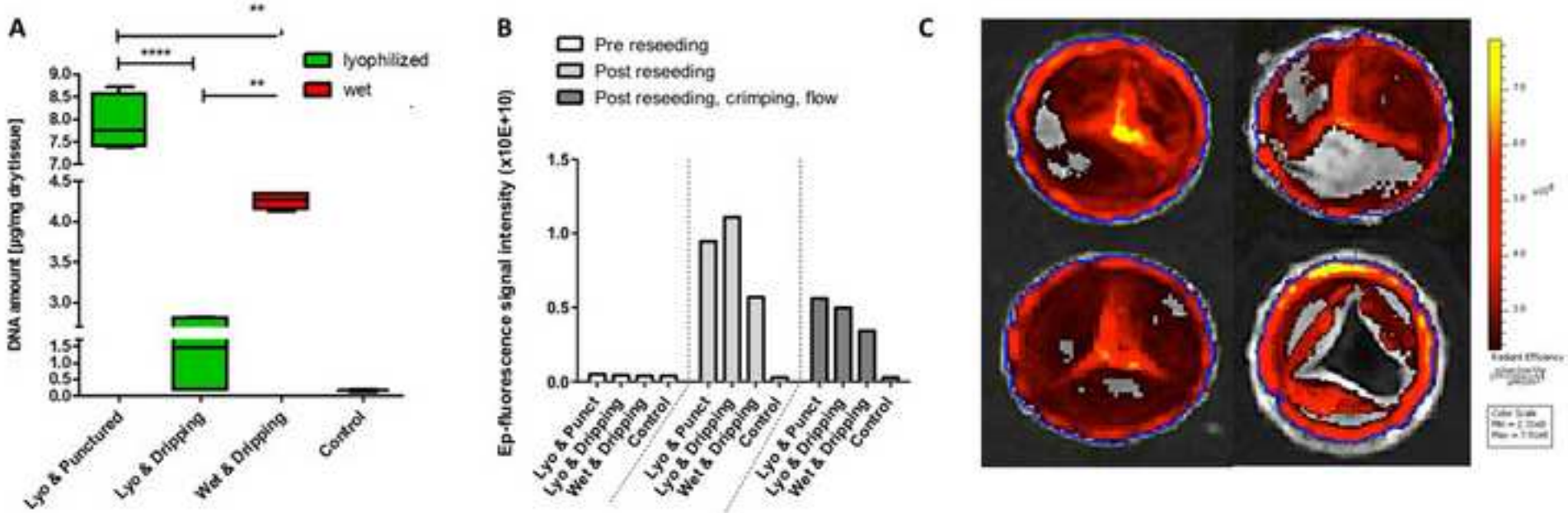
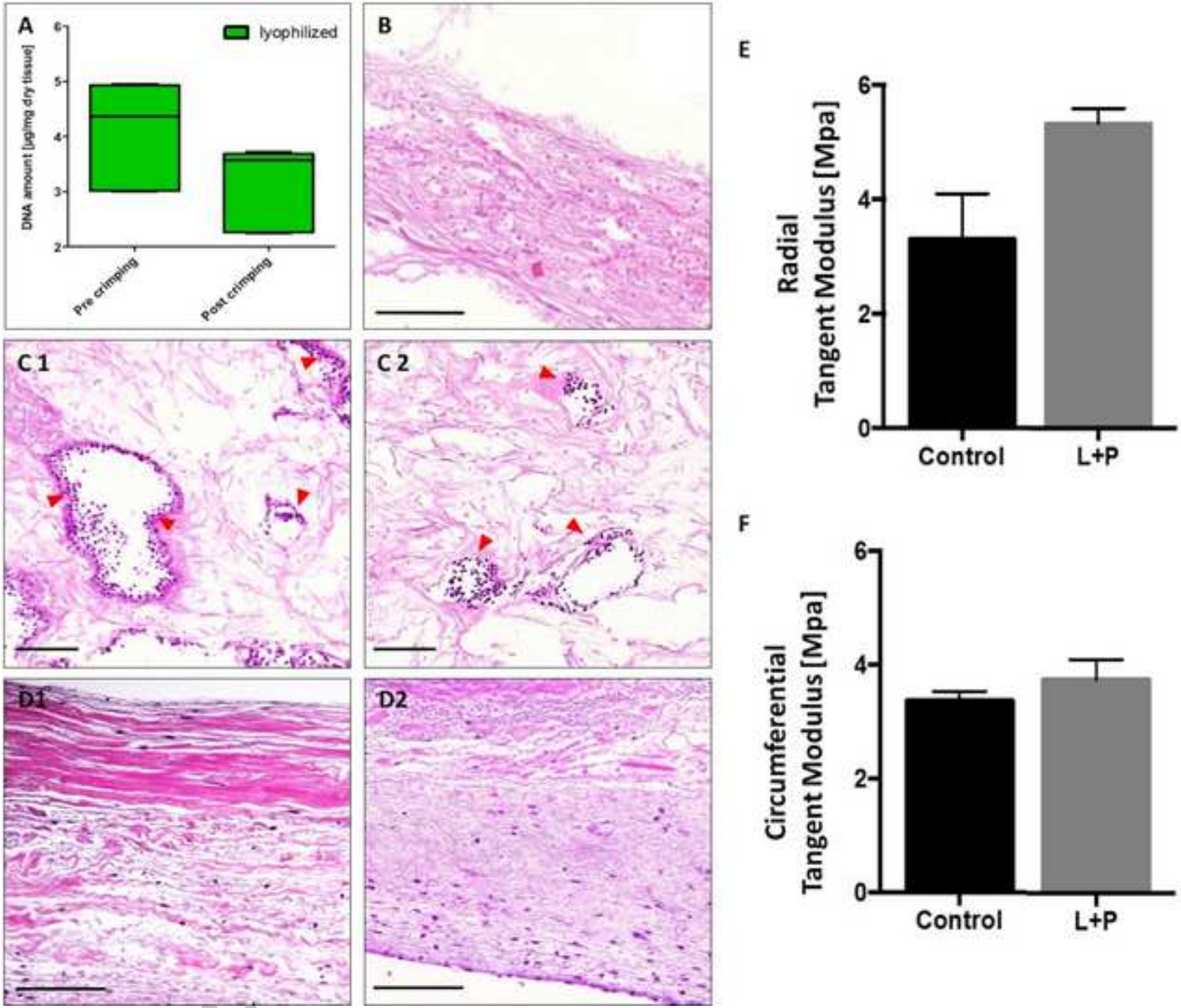
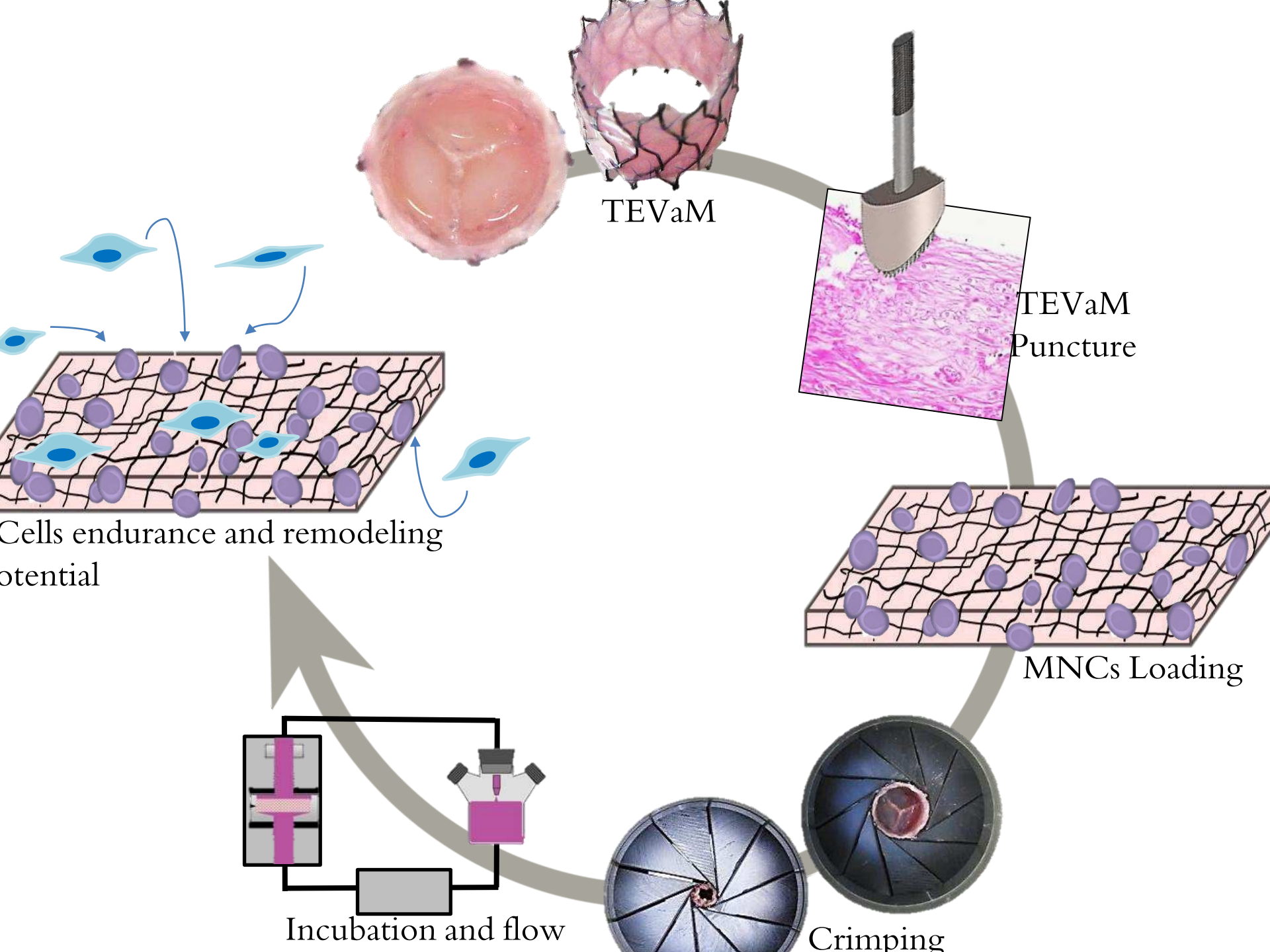


Figure 7






TEVaM
TEVaM
Puncture
MNCs Loading
Crimping
Incubation and flow
Cells endurance and remodeling
otential

Statement of significance

**Manuscript Title: Puncturing of lyophilized tissue engineered vascular matrices enhances the efficiency of their recellularization.**

The concept of living tissue engineered, self-repairing, autologous cardiovascular replacements, was proposed alternatively to existing synthetic/xenogeneic prostheses. Recent studies in animal models demonstrate faster *in vivo* recellularization after grafts pre-seeding with cells prior implantation. Pre-seeded cells hold either, the ability to differentiate directionally or attract host cells, crucial for graft integration and remodeling. It is unclear, however, how efficient the pre-loading is and how well cells withstand the flow. The study presents a systematic overview on cell loading techniques of different cardiovascular constructs, tested under static and dynamic conditions. Comparison illustrates a significantly higher efficiency of cells loading in lyophilized tissues punctured before their standard seeding. This technique may beneficially accelerate remodeling of cardiovascular grafts in further *in vivo* studies.